Institut für Veterinärphysiologie
der Vetsuisse-Fakultät Universität Zürich

Direktor: Prof. Dr. M. Gassmann

Amylin and GLP-1 target different populations of area postrema neurons that are both modulated by nutrient stimuli

Inaugural-Dissertation

zur Erlangung der Doktorwürde der
Vetsuisse-Fakultät Universität Zürich

vorgelegt von

Daniela Züger

Tierärztin
von Altendorf, Schwyz

genehmigt auf Antrag von

PD Dr. Thomas Riediger, Referent

Zürich 2013

Contents

Vetsuisse Fakultät Universität Zürich (2013)

Daniela Züger

Institut für Veterinärphysiologie, sekretariat@vetphys.uzh.ch

Amylin and GLP-1 target different populations of area postrema neurons that are both modulated by nutrient stimuli

Die Area postrema (AP) vermittelt den Sättigungseffekt des Pankreas-Hormons Amylin und ist auch sensitiv für Glucagon-like-peptide-1 (GLP-1), welches unter gewissen Bedingungen die Nahrungsaufnahme über die AP hemmt. Gemessen anhand der c-Fos Expression, aktiviert Amylin die AP stärker in 24h gefasteten Ratten als in ad libitum gefütterten Ratten. Dies beruht auf einer Verminderung der Amylin Responsivität durch Proteine. In dieser Studie wurde untersucht, ob die GLP-1 induzierte AP-Aktivierung ebenfalls in 24h gefasteten Ratten erhöht ist. Anhand des Calcitonin Rezeptors (CTR) als Marker für Amylin sensitive Neurone, untersuchten wir weiter, ob Amylin und GLP-1 dieselben AP Neurone aktivieren. Dies wurde auch für den Aversionsstimulus LiCl und für Angiotensin II (AngII) getestet, welches über die AP Herz-Kreislauf-Funktionen beeinflusst. GLP-1 induzierte nur in gefasteten aber nicht in ad libitum gefütterten Ratten eine deutliche c-Fos Antwort. Im Gegensatz zur hohen immunhistologischen Kolokalisation von Amylin induziertem c-Fos und CTR (68%), trat keine Kolokalisation auf nach Behandlung mit GLP-1, dem GLP-1R Agonist Exendin-4, LiCl oder AngII. Die Nahrungsaufnahme beeinflusst nicht nur die Responsivität der AP gegenüber Amylin, sondern auch gegenüber GLP-1. Amylin's Zielzellen unterscheiden sich von Neuronen, die durch GLP-1 aktiviert werden und durch Stimuli, die Aversion vermitteln oder kardiovasuläre Effekte haben. Amylin und der GLP-1 Rezeptor-Agonisten gelten als mögliche Behandlungsansätze gegen Fettleibigkeit und Diabetes. Daher könnte die Beeinflussung der neuronalen Responsivität durch Nahrungsstimuli eine klinische Relevanz für die Wirksamkeit derartiger Behandlungen haben.

Fütterungsaufnahme, Glucagon-like-peptide-1 Rezeptor, Area Postrema

Vetsuisse Fakultät Universität Zürich (2013)

Daniela Züger

Institut für Veterinärphysiologie, sekretariat@vetphys.uzh.ch

Amylin and GLP-1 target different populations of area postrema neurons that are both modulated by nutrient stimuli

The area postrema (AP) mediates the hypophagic effect of the pancreatic hormone amylin and is also sensitive to glucagon-like peptide-1 (GLP-1), which at least under some conditions inhibits feeding via the AP. Amylin activates the AP as measured by c-Fos expression more in 24h fasted than in ad libitum fed rats; the latter effect is due to an attenuation of the amylin responsiveness by diet-derived protein. It was aim of this study to investigate whether the GLP-1-induced AP activation is also increased in 24h fasted rats. Using the calcitonin receptor (CTR) as a marker for amylin sensitive neurons, we also investigated whether amylin and GLP-1 activate the same or different subsets of AP neurons. This was also tested for the aversive stimulus LiCl and for AngII, which modulates cardiovascular function via the AP. GLP-1 elicited a significant c-Fos response only in fasted but not in ad libitum fed rats. In contrast to a high cellular co-localization of amylin-induced c-Fos expression and CTR immunoreactivity (68%), no co-localization occurred between CTR and c-Fos immunoreactivity induced by GLP-1, the GLP-1R agonist exendin-4, LiCl or AngII. In conclusion, these findings indicate that the feeding status not only modulates AP responsiveness to amylin but also to the stimulatory effect of GLP-1 on AP neurons. Amylin's target cells seem to be different from neurons activated by GLP-1 or by stimuli involved in aversion or cardiovascular function. Because amylin and GLP-1 receptor agonists are in the focus of clinical trials identifying possible anti-obesity and anti-diabetes treatments, a modulation of neuronal responsiveness by nutritional stimuli might bear clinical relevance for the effectiveness of such treatments.

Food intake, glucagon-like-peptide-1 receptor, area postrema

Amylin and GLP-1 target different populations of area postrema neurons that are both modulated by nutrient stimuli

Daniela Züger *, Karoline Forster *, Thomas A. Lutz, Thomas Riediger

Institute of Veterinary Physiology and Centre of Integrative Human Physiology, University of Zurich, 8057 Zurich, Switzerland

Address of correspondence:
Thomas Riediger
Institute of Veterinary Physiology, University of Zurich
Winterthurerstrasse 260
8057 Zurich, Switzerland
Phone: +41-44-635-8815
Fax: +41-44-635-8932
e-mail: triedig@vetphys.uzh.ch

* These authors contributed equally to this work

Abstract

The area postrema mediates the hypophagic effect of the pancreatic hormone amylin and is also sensitive to glucagon-like peptide 1 (GLP-1). Protein seems to modulate amylin responsiveness because amylin seems to produce a stronger hypophagic effect and a stronger c-Fos expression when protein is absent from the diet. Accordingly, amylin induces a stronger c-Fos expression in the AP when injected in fasted compared to ad libitum fed rats. Here we tested the hypothesis that diet-derived protein attenuates the amylin dependent suppression of feeding and AP activation using isocaloric diets that differed in their protein content. Moreover, we investigated whether peripheral amino acid injection attenuates amylin-induced c-Fos expression in fasted rats. Since recent evidence suggests that GLP-1 may also reduce eating via the AP we tested whether 24h fasting also increases neuronal AP responsiveness to GLP-1 similar to the fasting-induced increase in amylin responsiveness. Finally, we used the calcitonin receptor (CTR) as an immunohistochemical marker for amylin-receptive AP neurons to investigate whether amylin's target neurons differ from GLP-1 responsive AP neurons. We also dissociated amylin responsive cells from neurons implicated in other AP-mediated functions such as aversion or blood pressure regulation. For this purpose we conducted c-Fos/CTR double staining after LiCl or angiotensin II treatment, respectively. Amylin (5 μg/kg sc) was more effective to reduce the intake of a 1% vs. an 8% or 18% protein diet and to induce c-Fos expression in the AP in rats receiving 1% vs. 18% protein diet. Increased protein intake was associated with increased blood amino acid levels. Peripheral injection of amino acids (1g/kg ip) attenuated the amylin-induced AP activation in 24h fasted rats. Similar to amylin, GLP-1 (100 μg/kg ip) elicited a significant c-Fos response only in fasted but not in ad libitum fed rats. However, in contrast to a high co-localization of amylin-induced c-Fos and CTR (68%), no c-Fos/CTR co-localization occurred after treatment

with GLP-1 or the GLP-1R agonist exendin 4 (2 μ g/kg ip). Similarly, LiCl (76mg/kg ip) or AngII (50 µg/kg sc) lead to c-Fos expression only in CTR negative AP neurons.

In conclusion, our findings support a protein-dependent modulation of behavioral and neuronal amylin responsiveness under equicaloric feeding conditions. Amino acids might contribute to the inhibitory effect of diet-derived protein to reduce amylin-induced neuronal AP activation. Neuronal AP responsiveness to GLP-1 is also increased in the fasted state suggesting that diet-derived nutrients may also interfere with AP-mediated GLP-1 effects. Nevertheless, the primary target neurons for amylin appear to be distinct from cells targeted by GLP-1 and by stimuli producing aversion (LiCl) or contributing to blood pressure regulation (AngII) via the AP. Since amylin and GLP-1 analogs are targets for the treatment of obesity, the nutrient-dependent modulation of AP responsiveness might entail implications for such therapeutic approaches.

Key words: food intake, glucagon-like peptide-1 receptor, exendin, brainstem, area postrema

1. Introduction

The pancreatic hormone amylin acts as a satiation signal via an excitation of area postrema (AP) neurons [1, 2]. Peripheral injection of amylin induces a c-Fos expression in noradrenergic AP neurons. Similar to a complete AP ablation [3], a specific chemical lesion of noradrenergic AP neurons blocks amylin's hypophagic affect [4]. The functional amylin receptor consists of the calcitonin receptor (CTR) as core component co-expressed with receptor activity modifying protein 1 or 3 (RAMP1, RAMP3) [5, 6]. The CTR and RAMP3 are expressed in the AP [7, 8]. We recently demonstrated that amylin induces a stronger c-Fos expression in 24h fasted vs. ad libitum fed rats [9]. This effect appeared to depend on diet-derived protein because the amylin-induced c-Fos expression was attenuated when rats were fed a non-caloric diet supplemented with protein while glucose or fat supplementation did not affect the amylin response [9].

In our current work we extended these studies by using isocaloric diets with different protein content (1%, 8%, 18%) in order to minimize the possible influence of the diet's caloric density. Differences in protein intake are likely to result in altered levels of circulating amino acids, which might exert a direct or indirect signaling function modulating the central amylin sensitivity. Due to the lack of a functional blood-brain-barrier in the AP, blood-borne amino acids might directly act on AP neurons. In order to assess the possible role of circulating amino acids in the modulation of the responsiveness of AP neurons to amylin we investigated whether the amylin-induced c-Fos response in the AP of fasted rats is attenuated by intraperitoneal administration of amino acids.

The AP may also represent a target structure for glucagon-like peptide 1 (GLP-1). GLP-1 is released by L-cells in response to nutrient stimuli [10, 11]. In addition GLP-1 can also be released locally in the AP from GLP-1ergic nerve terminals of enteroceptive neurons located in the nucleus of the nucleus of the solitary tract (NTS) [12, 13]. GLP-1 receptor (GLP-1R)

mRNA is highly abundant in the AP and peripherally injected radiolabled GLP-1 binds to these receptors [12, 14]. Both exogenous GLP-1 and the GLP-1R agonist exendin-4 induce a c-Fos response in the AP [15] and both peptides reduce meal size in rats [16]. The AP mediates the hypophagic effect of GLP-1 infused into the hepatic portal vein [17]. Due to the similarities between amylin and GLP-1 we hypothesized that neuronal GLP-1 responsiveness of the AP might also be affected by the feeding status. To test this we compared the GLP-1-induced c-Fos expression in fasted vs. ad libitum fed rats.

The AP is not only involved in the physiological control of eating. It also has a crucial function as a so-called chemoreceptor trigger zone for the sensing and processing of aversive and nausea-inducing stimuli [18]. Unlike amylin [1, 19], GLP-1 plays a role in the induction of nausea and conditioned taste aversion [20]. Interestingly, c-Fos expression induced in the AP by the aversive stimulus lithium chloride (LiCl) is mediated by GLP-1 [21]. The divergence in the aversive responses induced by amylin and GLP-1 suggests the existence of discrete subpopulations of AP neurons that are targeted by amylin or GLP-1, respectively. Assuming that amylin-responsive AP neurons express the CTR we conducted immunohistological CTR/c-Fos double staining studies in order to analyze whether GLP-1 or its agonist exendin-4 activate AP neurons that differ from cells that are activated by amylin. As a commonly used model for aversion we also included LiCl-treated animals in these studies. Furthermore, we used angiotensin II (AngII) as an additional functional stimulus because AngII is thought to primarily modulate the autonomic control of blood pressure but not feeding behavior via the AP [22].

2. Materials and Methods

2.1 Animals and housing conditions

Male Wistar rats (Elevage Janvier, Le Genest-St-Isle, France) were used in all experiments. Their mean body weight was 250g at the start of the experiments. The rats were adapted to the housing conditions and to handling for 10 days before the experiments started. The animals had ad libitum access to tap water and to standard laboratory rodent chow (#3430, 18.5% protein; Provimi Kliba, Gossau, Switzerland) except during periods when the animals received specific test diets or during food deprivation as described below. All rats were individually housed in hanging, stainless steel wire cages (50 x 25 x 18 cm) under controlled conditions of illumination (12:12 h light – dark cycle), humidity and temperature (21 ± 1°C). In some experiments isocaloric custom-made diets (Provimi Kliba AG; Kaiseraugst, Switzerland) were used which differed in their protein (18%, 8%, 1%) but not fat content. Caloric density (15.1 -15.4 MJ/kg) was adjusted by variable contents of carbohydrates (mainly starch). With marginal differences the 18% protein diet had a nutrient composition that corresponded to the standard chow. The Veterinary Office of the Kanton Zurich, Switzerland, approved all animal procedures and experiments.

2.2 Effect of diet-derived protein on amylin-induced hypophagia and c-Fos expression in the AP

In order to adapt the animals to the respective test diets, the 1%, 8%, or 18% protein diets were offered on 2 single days during a 7-10 day habituation period. Before the feeding trials that were conducted in intervals of 5 days, the animals received regular chow for 3 days, chow in combination with the respective test diet for one day and only the test diet for another day. At dark onset, amylin (5µg/kg s.c.; Bachem, Bubendorf, Switzerland) or saline (controls)

were injected; for the feeding tests, treatments were conducted in a crossover design for each diet in weight-matched groups. Cumulative food intake was measured 30 min, one hour and two hours after injection with a precision of 0.1g and correction for spillage. The animals were kept on the diet until 24h after the injection and then switched back to chow. The amylin-induced c-Fos expression was investigated in separate groups of rats fed 1% vs. 18% protein diet. The adaptation of the animals and the treatments were the same as for the feeding trials.

2.3 Effect of amino acids on amylin-induced c-Fos expression in the AP

To test the effect of circulating amino acids on the amylin-induced c-Fos expression in the AP the amino acid solution Aminoven (Fresenius Kabi Schweiz AG, Oberdorf, Switzerland) was used. Aminoven contains 16 different amino acids at a concentration of 15% and is used for medical purposes in parenteral nutrition. Four weight-matched groups of rats including a saline control group (n=5), an amylin-treated group (n=7), an Aminoven control group (n=8) and a group that was treated with Aminoven and amylin (n=7) were used.

The animals were food-deprived for 24 h and injected with Aminoven (1g/kg i.p.) or saline 20 min before dark onset. At dark onset, amylin (5 μg/kg s.c.) or saline were injected. Two hours after these treatments, the animals were deeply anaesthetized and transcardially perfused with ice-cold phosphate buffer solution (PB 0.1 M, pH 7.2) followed by 4% paraformaldehyde solution (4% PB 0.1 M) for fixation. Blood was sampled and the glucose concentration was measured as described below. The immunohistological detection of c-Fos is described in section 2.5.

2.4 Measurements of blood glucose, amino acids and amylin levels

To measure the effects of the different protein diets on blood glucose and amino acid levels, blood samples were taken at dark onset from the lingual vein under isoflurane aneasthesia. The experimental conditions were the same as in the respective feeding trials and c-Fos studies. Immediately after blood sampling, the concentration of glucose was measured using a glucose-oxidase based glucometer (Glucometer Elite; Bayer, Zürich).

The blood was then transferred into serum tubes (Microvette 500 Z, Sarstedt, Germany) and centrifuged at 2800 rpm for 10 min. A protease inhibitor mixture (P 2714, Sigma - Aldrich) was immediately added after blood sampling (10µl of the inhibitor / 1ml blood). The serum was then removed and frozen at -20 °C. The Functional Genomics Centre at the University of Zurich performed the measurement of amino acid concentrations. 80µl of each serum sample were used and 80µl of a 10% sulfasalicyclic acid solution was added for precipitation. 20µl of the supernatant was taken and 60µl borate buffer were added to the vial and reconstituted with 20µl MassTrak Amino Acid Analysis Solution derivatization reagent. 1µl of this vial was injected into the Waters Acquity Ultra Performance Liquid Chromatography (UPLC) machine equipped with an UV detector (Waters Corporation, Milford, MA, USA) and the samples were analyzed. Analyses were carried out according to the manufacturer's instructions.

2.5 Immunohistological studies

c-Fos single staining

Single staining c-Fos studies were conducted to investigate the effects of fasting on GLP-1 and AngII-induced AP activation. Animals were food deprived for 24h before receiving GLP-1 or AngII at dark onset (100µg/kg or i.p. or 50µg/kg s.c., respectively; both from Bachem, Bubendorf, Switzerland). Similar to our previous studies [9], a non-fluorescent approach was used for the detection of c-Fos.

Sections were incubated with a polyclonal rabbit anti-c-Fos antibody (Ab-5, Calbiochem, Darmstadt, Germany) in 0.3% PBST (1:5000). After washing in 0.1% PBST (5 x 10 min), the brain sections were incubated with the secondary antibody (1:400 biotinylated goat anti-rabbit antibody; Vector Laboratories, Burlingame, California USA) for 90 minutes at room temperature. For the diaminobenzidine staining a Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, California USA) was used. The staining procedure was identical to the methods described in our previous studies [9]. For c-Fos studies conducted to test the effects of the diet's protein content and amino acid treatment on the amylin-induced AP activation the same method was used.

c-Fos stainings were evaluated using a microscope equipped with a digital camera system (Axioskop; Carl Zeiss, Feldbach, Switzerland). The experimenter was blinded to the experimental groups. c-Fos positive cells were counted in three sections per animal at the mid rostro-caudal level of the AP (approximately at bregma -14.04; [23]). The cell counts were averaged for each animal. In order to correct for interassay variability appropriate control groups were included in all experiments. Group means ± SEM was calculated from the averaged cell counts of each animal for each treatment group (n = number of animals).

c-Fos/CTR double staining

The co-localisation of c-Fos and CTR expression was investigated in 24h fasted rats that received the following injections at dark the onset: amylin (5µg/kg s.c), GLP-1 (100µg/kg i.p), exendin-4 (2µg/kg i.p.; Bachem, Bubendorf, Switzerland), LiCl (76mg/kg i.p.; Aldrich, Buchs SG, Switzerland), AngII (50µg/kg s.c.) or saline as control. Two hours after injections, the animals were deeply anaesthetized with pentobarbital sodium (100 mg/kg i.p.) and perfused as described above. The brains were removed and postfixed for additional 2h in a 4% paraformaldehyde solution at 4 °C. After incubation for 48h in 20 % sucrose in PB for cryoprotection, the brains snap frozen with CO_2.

Coronal sections (20 µm) of the medulla oblongata at the level of the AP (bregma -14.28mm to -13.68mm; [23]) were cut in a cryostat (Leica CM 3050 S, Nussloch, Germany), thaw-mounted on adhesive glass slides (Super Frost Plus; Menzel Braunschweig, Germany) and stored at -20°C. After 1h air drying, the sections were rehydrated in phosphate buffered saline (PBS pH 7.4) for 10min. The slides were incubated with normal donkey serum (1.5% in PBS containing 0.3% Triton X100; PBST) for 2h. After 30min of washing, two primary antibodies were co-applied in 0.3% PBST for 48h at 4°C: rabbit anti-CTR (1:1000; kindly provided by P. Wookey, University of Melbourne, Australia [24]) and goat anti-c-Fos (1:500; sc-52-G, Santa Cruz). After washing for 50 minutes, slices were incubated with two secondary antibodies: donkey anti-rabbit Alexa-555 (1:100) and donkey anti-goat Alexa-488 (1:200) for two hours at room temperature (both from Invitrogen, Basel, Switzerland). The sections were rinsed for 30min in 0.1% PBST 0.1% and PBS and then covered with citifluor (Citifluor Ltd., London, UK).

In similar immunohistochemical double staining experiments the co-localization of amylin-induced formation of the second messenger cyclic guanosine monophosphate (cGMP) and CTR was investigated (see supplementary material).

2.6 Statistical Analysis

All results are presented as mean values ± SEM. Statistical comparisons across more than 2 experimental groups were conducted by one way ANOVA or one way ANOVA on ranks (Kruskal-Wallis test) for data that were not normally distributed, followed by the Student-Newman-Keuls or Dunn's multiple comparison post hoc test, respectively. In the immunohistological studies, the mean value of the cell counts/section of an individual animal was used for statistical analyses. Differences in food intake were analyzed using a paired

Student's *t*-test to compare the differences between amylin-treated and their control groups at each time point. In all cases $p < 0.05$ was considered significant.

3. Results

3.1 Modulation of hormonal responsiveness by nutrient stimuli

Baseline intake of the isocaloric diets that differed in protein content was measured over 24h. While rats showed similar baseline intake of the 1% and 18% protein diet, the animals ate slightly but significantly less of the 8% protein diet (figure 1A). The protein content of the diets affected the level of total blood amino acids measured 2h after presentation of the diet. Rats receiving the 18% protein diet showed blood amino acid levels that were similar to the level observed in rats receiving standard chow. Intake of the 1% and 8% protein diet resulted in significantly lower concentration of circulating amino acids (figure 1B). There were no differences in the blood glucose levels between three groups (figure 1C).

Amylin significantly reduced food intake across all groups, i.e. in rats fed the 1%, the 8% or the 18% diet. The amylin-induced reduction in food intake was significant after 30 min and 60 min under all three feeding conditions (figure 2ABC). After 120 min, amylin only reduced intake in rats fed the 1% protein diet, but not the 8% or 18% protein diets. When compared to the pertinent control groups, amylin caused a stronger relative suppression of 30 and 60 min food intake in rats fed the 1% protein diet than in animals receiving the 8% or 18% protein diets (figure 2D).

To investigate the influence of the diet-derived protein on the amylin-induced AP activation, amylin-induced c-Fos expression was compared in rats fed the 1% or 18% protein diet. There was no difference in c-Fos expression between rats fed 18% fed and 1% in the saline-treated

controls. The amylin-induced c-Fos response in the AP of animals that were fed the 18% diet was significantly lower than in animals fed the 1% diet (figure 3).

The amylin-induced AP activation was also attenuated by intraperitoneal administration of amino acids (Aminoven) in 24h fasted rats. Aminoven injection alone did not alter c-Fos expression relative to saline treated controls because both groups showed only little c-Fos expression. Although amylin induced a significant c-Fos response in the AP of the Aminoven/amylin treated animals, the number of c-Fos expressing cells in the AP was significantly lower than in the saline/amylin treated group (figure 4). Notably, the stronger AP activation in the latter food-deprived group relative to the ad libitum fed 18% protein diet group shown in figure 3 is due to the well-described effect of fasting on amylin-induced c-Fos expression [9].

Proceeding from the observation that fasting increases amylin-induced AP activation, we investigated whether the responsiveness of AP neurons to GLP-1 or AngII is also stronger after 24h fasting. While GLP-1 failed to induce a c-Fos response under ad libitum conditions, it induced a significant AP activation in 24h fasted rats (figure 5A). The AngII-induced c-Fos responses were weak. AngII elicited a significant AP activation in chow fed animals but not in fasted rats, although the mean number of c-Fos positive neurons was similar and not significantly different across the two feeding conditions (figure 5B).

3.2 Immuohistochemical phenotyping studies (c-Fos/CTR double staining)

Amylin treatment elicited a pronounced c-Fos response in the AP while under control conditions only few AP neurons were c-Fos positive (Figure 6A-C). In line with the assumption that CTR-expressing AP neurons are targeted by amylin, the majority of amylin-

activated cells were CTR positive; in the amylin-treated group 68% of all c-Fos positive neurons showed CTR immunoreactivity while no c-Fos/CTR immunoreactive cells were detected in the saline treated control group (Figure 6C). As previously described [7] the CTR was mainly localized in the cell membranes. A representative immunostaining demonstrating the co-localization between amylin-induced c-Fos expression and CTR is shown in figure 6A (see figure 6B for higher magnification).

GLP-1R stimulation with exendin-4 or GLP-1 significantly increased c-Fos expression in the AP (Figure 6D-F and 6J, respectively). Interestingly, both exendin-4 and GLP-1 activated AP neurons that did not express the CTR. Only 2-3% of all cells expressing c-Fos after exendin-4 (Figure 6F) or GLP-1 treatment (Figure 6J) were CTR positive indicating that GLP-1R stimulation activates a population of AP neurons that appears to be largely distinct from the direct target cells for amylin.

LiCl induced a strong c-Fos response in the AP. Similar to exendin-4 and GLP-1, LiCl predominantly activated CTR negative AP neurons because only 3% of the c-Fos positive AP neurons expressed the CTR after LiCl treatment (Figure 6K). AngII induced a moderate but significant c-Fos response in the AP. As shown in figure 6G-I hardly any of the c-Fos expressing cells were CTR-positive (1%) after treatment with AngII.

4. Discussion

4.1 Modulation of hormonal responsiveness by nutrient stimuli

As suggested by our previous studies [9] diet-derived protein seems to attenuate amylin-sensitivity. These previous studies however did not account for possible effects that might be related to differences in caloric intake. Furthermore the previous studies were conducted under artificial feeding conditions because the diets only contained single nutrients. We now

show that amylin`s hypophagic action was much stronger in rats fed the 1% protein diet compared to the 8% and 18% diet. To test whether the attenuation of amylin's hypophagic action is paralleled by a reduced amylin-mediated AP activation, the effects of amylin on c-Fos expression were compared in rats fed the 1% vs. the 18% protein diet. In line with the observations of the behavioral experiments, amylin induced a lower c-Fos response in animals receiving the 18% protein diet. The excitatory action of amylin on AP neurons is considered the primary neuromechanism underlying amylin's feeding inhibitory effect. It is therefore conceivable that the impact of diet-derived protein on amylin's hypophagic action is mediated by an attenuation of the amylin-responsiveness of AP neurons.

It might appear unexpected that despite a low c-Fos response, amylin produced a significant hypophagic response in rats receiving the 18% protein diet. We observed similar results in previous studies [9] conducted with nutritionally equivalent standard chow. Although c-Fos is an accepted and useful marker for neuronal activation, the thresholds for amylin-induced c-Fos expression, amylin-induced neuronal activation and amylin's effects on feeding do not necessarily correspond.

Interestingly, amylin's hypophagic effect was similar in rats receiving the 8% and 18% protein diets. Therefore, dynamic effects of the protein content on amylin responsiveness seem to occur at protein contents of the diet lower than 8% at least when animals are fed ad libitum. Unlike the baseline intakes of the different protein diets in our short-term experiments, previous studies have demonstrated reduced intake of 2% protein diets under chronic feeding conditions [25]. The mechanism behind the reduced low protein intake during chronic exposure has not been identified. Taking our current findings into account, it is tempting to speculate that an increased responsiveness to endogenous satiation signals might have contributed to the reduced low protein intake reported in these studies.

Notably, under free living or non-laboratory conditions, protein supply is likely to vary, both with respect to the frequency of intake (food availability) and in terms of the diet's protein content. For these reasons short-term excursions in protein supply might modulate amylin-responsiveness under natural feeding conditions. In order to mimic such short-term excursions in food availability we investigated amylin-induced c-Fos expression in rats that were food-deprived for only 1h or 5h before dark onset. Amylin induced only a weak but non-significant c-Fos response in ad libitum fed animals (see supplementary material Figure 8A). In contrast, amylin-induced c-Fos expression was markedly increased in 5h but not 1h food deprived rats relative to ad libitum fed animals. These observations support the assumption that also comparatively moderate restrictions in food availability increase neuronal amylin responsiveness.

According to their protein contents, the diets used in the current study produced significantly different levels of blood amino acids (highest value for 18% diet). Hence, a postabsorptive effect of circulating amino acids might be relevant for the modulation of amylin-sensitivity under the different diet conditions that we tested. The attenuation of amylin-induced c-Fos expression in the AP of fasted rats by peripherally injected amino acids seems to confirm this hypothesis. The current findings may suggest a direct action of amino acids on amylin-responsive AP neurons. It also appears plausible that secondary hormonal effects might be involved because an increase in circulating blood amino acids represents a stimulus for gastrointestinal and pancreatic hormones; however, to study such effects was beyond the scope of the present study.

Based on the current results is appears unlikely that diet-dependent differences in blood glucose potentially induced by a different gluconeogenic activity of the diets might have had an influence on the effects of amylin. This issue is of particular interest because amylin-sensitive neurons have been shown to be glucose-responsive [26, 27]. Furthermore, amylin's

inhibition of gastric emptying, which also depends on the AP, is overridden by hypoglycemia [28]. Such effects most likely do not account for the differences in amylin signaling observed in the present study because there were no differences in glucose levels among the diet groups.

Interestingly, we also demonstrated an increased AP responsiveness for GLP-1 but not for AngII after 24h fasting compared to ad libitum feeding conditions. Based on the proposed function of the AP as a possible target site mediating a satiating effect of GLP-1 [17], a modulation of responsiveness by the feeding status might have a similar relevance for amylin and GLP-1, although the target neurons for these hormones do not seem to be identical. Studies directly investigating the influence of the feeding status on GLP-1 mediated effects are scare. As demonstrated by in vivo imaging techniques GLP-1 activates the AP in fasted mice but this effect has not been compared to ad libitum fed animals [29].

4.2 Immuohistochemical phenotyping studies (c-Fos/CTR double staining) and functional implications

Consistent with the notion that the CTR represents the core unit of the functional amylin receptor [5, 30], Our immunohistological double staining studies clearly indicate that CTR expressing AP neurons are major target cells for amylin. The fact that not all amylin-activated AP neurons were CTR positive might be explained by an indirect activation of CTR negative second-order neurons via AP intrinsic synaptic connections. This assumption is supported by our observation of an almost complete co-localization (96%) between amylin-induced cGMP formation and CTR expression in the AP (see supplementary material). cGMP is considered to be the intracellular second messenger mediating the amylin-induced excitation of AP

neurons [2]. Therefore, cGMP formation occurs specifically in the first-order target cells for amylin but not necessarily in synaptically activated neurons, which is in line with the higher degree of co-localization for amylin-induced cGMP/CTR vs. c-Fos/CTR.

The high overlap between amylin-induced c-Fos expression and CTR immunoreactivity contrasts with the absence of activated CTR positive AP neurons after GLP-1R stimulation elicited by GLP-1 or exendin-4. This finding suggests the existence of distinct populations of primary target cells in the AP for GLP-1 and amylin. It cannot be excluded, however, that excitatory amylin signaling indirectly converges on AP neurons that are also activated by GLP-1. Exendin-4, LiCl and amylin have been shown to activate catecholaminergic AP neurons [4, 31, 32]. Our current findings add to the existing studies by demonstrating a clear dissociation between the putative target cells for amylin and neurons that are activated by GLP-1 agonists or LiCl. Based on the existing phenotyping studies our current results might imply that CTR positive neurons that are activated by amylin represent a subset of catecholaminergic AP neurons. To our knowledge there are currently no published studies investigating the neurochemical phenotype of CTR versus non-CTR expressing AP neurons.

The role of the AP with respect to GLP-1R mediated functions is less understood than AP-dependent actions of amylin. This particularly applies to the question whether GLP-1 exerts a specific satiating effect via the AP or whether GLP-1 signaling in the AP primarily mediates aversive sensations such as emesis or nausea. Despite the ability of GLP-1 to produce symptoms of visceral illness it has been put forward that different neuronal systems mediate GLP-1-induced aversion and specific hypophagic effects. This is supported by the induction of anorexia with or without CTA following site-specific central injections of GLP-1. Interestingly, fourth ventricular infusion of GLP-1 inhibits feeding at doses that do not cause a CTA suggesting a possible involvement of hindbrain GLP-1R in the specific control of food intake [20]. Notably, our demonstration that GLP-1R activation does not activate CTR

positive AP neurons does not exclude the possibility that GLP-1 or exendin-4 stimulate AP neurons that are specifically involved in the control of feeding behavior. If so, however, these cells are most likely distinct from AP neurons that are directly targeted by amylin but they might overlap with a neuronal population of AP neurons that could be synaptically activated by amylin signaling.

Recent studies seem to imply that the experimental conditions add substantial complexity to the investigation of possible AP-dependent effects of GLP-1 on feeding behavior. While after hepatic portal vein infusion the hypophagic effect of peripherally applied GLP-1 is blunted by an AP lesion [17], the effect of intraperitoneally administered exendin-4 is not blocked in AP lesioned rats; of note, native GLP-1 was not tested in the latter series of experiments [33]. Whether the AP-dependent feeding suppressive effect of GLP-1 was related to an aversive action in these studies has not been elucidated. In contrast to amylin, GLP-1 and exendin-4 are known to induce emesis and nausea in humans [34, 35] and to induce aversive effects in rodents that are reflected by the ability of these peptides to trigger a conditioned taste aversion (CTA) [20]. Based on our current findings the differential aversive properties of amylin vs. GLP-1/exendin-4 might be attributed to the activation of different subsets of AP neurons.

The role of the AP in mediating aversive responses is well characterized. The development of a LiCl-induced CTA can be prevented by AP lesion [36] or cooling [37] indicating the importance of the AP for the processing of aversive stimuli. Our observation that LiCl-induced neuronal activation does not occur in the target cells for amylin strongly supports the idea that the amylin responsive population of AP neurons is not implicated in the perception or processing of stimuli producing malaise, which is in line with the lack of such responses after amylin treatment. While there seem to be largely discrete subsets of AP neurons responding to amylin on one hand and to GLP-1/exendin-4 or LiCl on the other hand, we didn’t aim to dissociate the neuronal populations responding to GLP-1R stimulation and LiCl.

As suggested by previous studies, these populations of neurons might overlap because the LiCl-induced c-Fos expression in the AP is blocked by central GLP-1R antagonism [21]. Hence, the LiCl-dependent activation of the AP and LiCl-induced CTA appears to be at least partly mediated by brain-intrinsic GLP-1, which has been confirmed in analogous behavioral studies [38].

We have used AngII as a further functional stimulus to dissociate amylin-sensitive cells from AP neurons that are involved in the cardiovascular control. The role of the AP as a central target site mediating cardiovascular effects of AngII is well known and numerous studies demonstrated the relevance of AP-dependent AngII effects on heart rate, blood pressure and baroreceptor reflex control in different species [39, 40]. The absence of AngII-induced c-Fos expression in CTR positive AP neurons underlines the segregation of amylin-sensitive neurons from the neuronal system implicated in cardiovascular control. This finding therefore contributes to the validation of our approach to use the CTR as a marker for amylin's target cells in the AP.

In conclusion our studies provide evidence that CTR expressing AP neurons represent the target cells for amylin in the AP and that these neurons are not activated by GLP-1R receptor activation, the aversive stimulus LiCl or AngII. Protein seems to modulate amylin sensitivity as reflected by an attenuation of amylin-induced AP activation and hypophagia. Amino acids appear to contribute to the attenuation of neuronal amylin responsiveness. These findings extend the concept that amylin signaling and amylin's ability to decrease food intake is influenced by diet-derived protein. A diet-dependent modulation of neuronal responsiveness in the AP also exists for GLP-1. Whether this effect is protein-dependent remains to be demonstrated. The attenuation of amylin and GLP-1 sensitivity by diet-derived nutrients might have therapeutic implications, because amylin and GLP-1 receptor agonists are in the

focus of clinical trials identifying possible anti-obesity and anti-diabetic treatments. Importantly, therapeutic approaches combining amylin and GLP-1 analogs yielded promising outcome in primate studies [41]. Based on our current findings the feeding status and particularly the intake of protein might have a substantial impact on the effectiveness of such treatments.

Supplementary material

As reported in our previous studies, amylin induced a strong c-Fos expression in the AP in 24h fasted rats [9]. To evaluate the effect of shorter periods of food deprivation on amylin sensitivity of AP neurons, we investigated amylin-induced c-Fos expression in rats after 5h and 1h food deprivation. Amylin induced only a weak but non-significant c-Fos response in ad libitum fed animals (supplementary figure 1). In contrast, amylin markedly increased c-Fos expression in 5h food deprived rats. The number of activated AP neurons under these conditions was not only significantly higher than in the controls but differed also significantly from the mean c-Fos response in chow-fed/amylin-treated rats. Interestingly, 1h food deprivation was not sufficient to increase amylin the amylin responsiveness of the AP relative to ad libitum feeding conditions. Although the amylin-mediated c-Fos response was significant in 1h food deprived rats, it was almost identical and not significantly different from the AP activation observed in the ad libitum fed animals of this experiment.

As an independent immunohistological approach to confirm that CTR expressing AP neurons are targeted by amylin we conducted a CTR/cGMP double staining after amylin treatment. The antibody [42] and the protocol to detect cGMP formation were similar as published before [2]. Briefly, ad libitum fed animals were pre-treated with the phosphodiesterase

inhibitor 3-isobutyl-1-methyl-xanthin (IBMX, 1mg ip, Fluka, Buchs SG, Schweiz) 15 min before amylin or saline injection. For this study a higher dose of amylin (20µg/kg s.c.) has been used because cGMP is difficult to be detected at lower amylin doses probably due to the enzymatic degradation resulting from remaining phosphodiesterase activity. The immunohistochemical CTR staining was conducted after the cGMP staining according to the procedures described in the material and methods. A secondary donkey anti-rabbit, Cy3-conjugated antibody was used in a dilution of 1:225 (Milan Analytica, La Roche, Schweiz).

In line with our published work [2], cGMP positive neurons were absent or very few in control animals (n=3, data not shown). Supplementary figure 2 shows a detailed analysis of the cGMP/CTR co-localization in one of three amylin-treated rats. With only few exceptions almost all cGMP positive AP neurons (96%) expressed the CTR. A similar outcome was obtained for the two other amylin-treated rats (not shown).

Acknowledgements

The authors thank P. Wookey (University of Melbourne, Australia) for kindly providing the antibody against the calcitonin receptor.

References

[1] Lutz, T. A., Geary, N., Szabady, M. M., Del Prete, E., Scharrer, E. Amylin decreases meal size in rats. Physiol Behav. 1995,58:1197-202.

[2] Riediger, T., Schmid, H. A., Lutz, T., Simon, E. Amylin potently activates AP neurons possibly via formation of the excitatory second messenger cGMP. Am J Physiol Regul Integr Comp Physiol. 2001,281:R1833-43.

[3] Lutz, T. A., Senn, M., Althaus, J., Del Prete, E., Ehrensperger, F., Scharrer, E. Lesion of the area postrema/nucleus of the solitary tract (AP/NTS) attenuates the anorectic effects of amylin and calcitonin gene-related peptide (CGRP) in rats. Peptides. 1998,19:309-17.

[4] Potes, C. S., Turek, V. F., Cole, R. L., Vu, C., Roland, B. L., Roth, J. D., et al. Noradrenergic neurons of the area postrema mediate amylin's hypophagic action. Am J Physiol Regul Integr Comp Physiol. 2010,299:R623-31.

[5] Christopoulos, G., Perry, K. J., Morfis, M., Tilakaratne, N., Gao, Y., Fraser, N. J., et al. Multiple amylin receptors arise from receptor activity-modifying protein interaction with the calcitonin receptor gene product. Mol Pharmacol. 1999,56:235-42.

[6] Bailey, R. J., Walker, C. S., Ferner, A. H., Loomes, K. M., Prijic, G., Halim, A., et al. Pharmacological characterization of rat amylin receptors: implications for the identification of amylin receptor subtypes. Br J Pharmacol. 2012,166:151-67.

[7] Becskei, C., Riediger, T., Zund, D., Wookey, P., Lutz, T. A. Immunohistochemical mapping of calcitonin receptors in the adult rat brain. Brain Res. 2004,1030:221-33.

[8] Barth, S. W., Riediger, T., Lutz, T. A., Rechkemmer, G. Peripheral amylin activates circumventricular organs expressing calcitonin receptor a/b subtypes and receptor-activity modifying proteins in the rat. Brain Res. 2004,997:97-102.

[9] Michel, S., Becskei, C., Erguven, E., Lutz, T. A., Riediger, T. Diet-derived nutrients modulate the effects of amylin on c-Fos expression in the area postrema and on food intake. Neuroendocrinology. 2007,86:124-35.

[10] Kreymann, B., Williams, G., Ghatei, M. A., Bloom, S. R. Glucagon-like peptide-1 7-36: a physiological incretin in man. Lancet. 1987,2:1300-4.

[11] Orskov, C., Wettergren, A., Holst, J. J. Secretion of the incretin hormones glucagon-like peptide-1 and gastric inhibitory polypeptide correlates with insulin secretion in normal man throughout the day. Scan J Gastroenterol. 1996,31:665-70.

[12] Merchenthaler, I., Lane, M., Shughrue, P. Distribution of pre-pro-glucagon and glucagon-like peptide-1 receptor messenger RNAs in the rat central nervous system. J Comp Neurol. 1999,403:261-80.

[13] Jin, S. L., Han, V. K., Simmons, J. G., Towle, A. C., Lauder, J. M., Lund, P. K. Distribution of glucagonlike peptide I (GLP-I), glucagon, and glicentin in the rat brain: an immunocytochemical study. J Comp Neurol. 1988,271:519-32.

[14] Orskov, C., Poulsen, S. S., Moller, M., Holst, J. J. Glucagon-like peptide I receptors in the subfornical organ and the area postrema are accessible to circulating glucagon-like peptide I. Diabetes. 1996,45:832-5.

[15] Kanoski, S. E., Fortin, S. M., Arnold, M., Grill, H. J., Hayes, M. R. Peripheral and central GLP-1 receptor populations mediate the anorectic effects of peripherally administered GLP-1 receptor agonists, liraglutide and exendin-4. Endocrinology. 2011,152:3103-12.

[16] Scott, K. A., Moran, T. H. The GLP-1 agonist exendin-4 reduces food intake in nonhuman primates through changes in meal size. Am J Physiol Regul Integr Comp Physiol. 2007,293:R983-7.

[17] Pacheco-Lopez, G., Punjabi, M., Graber, M., Geary, N., Arnold, M., Langhans, W. Hindbrain glucagon-like peptide-1 receptors (GLP-1R) mediate the eating-inhibitory effect of hepatic portal vein (HPV) GLP-1 infusions. Appetite. 2010,54:668.

[18] Tsukamoto, G., Adachi, A. Neural responses of rat area postrema to stimuli producing nausea. J Auton Nerv Syst. 1994,49:55-60.

[19] Morley, J. E., Suarez, M. D., Mattamal, M., Flood, J. F. Amylin and food intake in mice: effects on motivation to eat and mechanism of action. Pharmacol Biochem Behav. 1997,56:123-9.

[20] Kinzig, K. P., D'Alessio, D. A., Seeley, R. J. The diverse roles of specific GLP-1 receptors in the control of food intake and the response to visceral illness. J Neurosci. 2002,22:10470-6.

[21] Thiele, T. E., Seeley, R. J., D'Alessio, D., Eng, J., Bernstein, I. L., Woods, S. C., et al. Central infusion of glucagon-like peptide-1-(7-36) amide (GLP-1) receptor antagonist attenuates lithium chloride-induced c-Fos induction in rat brainstem. Brain Res. 1998,801:164-70.

[22] Joy, M. D., Lowe, R. D. Evidence that the area postrema mediates the central cardiovascular response to angiotensin II. Nature. 1970,228:1303-4.

[23] Paxinos, G., Watson, C. The rat brain in stereotaxic coordinates. Amsterdam; Boston: Academic Press/Elsevier; 2007.

[24] Tikellis, C., Xuereb, L., Casley, D., Brasier, G., Cooper, M. E., Wookey, P. J. Calcitonin receptor isoforms expressed in the developing rat kidney. Kidney Int. 2003,63:416-26.

[25] Du, F., Higginbotham, D. A., White, B. D. Food intake, energy balance and serum leptin concentrations in rats fed low-protein diets. J Nutr. 2000,130:514-21.

[26] Adachi, A., Kobashi, M., Funahashi, M. Glucose-responsive neurons in the brainstem. Obes Res. 1995,3 Suppl 5:735S-40S.

[27] Riediger, T., Schmid, H. A., Lutz, T. A., Simon, E. Amylin and glucose co-activate area postrema neurons of the rat. Neurosci Lett. 2002,328:121-4.

[28] Gedulin, B. R., Young, A. A. Hypoglycemia overrides amylin-mediated regulation of gastric emptying in rats. Diabetes. 1998,47:93-7.

[29] Parkinson, J. R., Chaudhri, O. B., Kuo, Y. T., Field, B. C., Herlihy, A. H., Dhillo, W. S., et al. Differential patterns of neuronal activation in the brainstem and hypothalamus following peripheral injection of GLP-1, oxyntomodulin and lithium chloride in mice detected by manganese-enhanced magnetic resonance imaging (MEMRI). Neuroimage. 2009,44:1022-31.

[30] Tilakaratne, N., Christopoulos, G., Zumpe, E. T., Foord, S. M., Sexton, P. M. Amylin receptor phenotypes derived from human calcitonin receptor/RAMP coexpression exhibit pharmacological differences dependent on receptor isoform and host cell environment. J Pharmacol Exp Ther. 2000,294:61-72.

[31] Rinaman, L., Dzmura, V. Experimental dissociation of neural circuits underlying conditioned avoidance and hypophagic responses to lithium chloride. Am J Physiol Regul Integr Comp Physiol. 2007,293:R1495-503.

[32] Yamamoto, H., Kishi, T., Lee, C. E., Choi, B. J., Fang, H., Hollenberg, A. N., et al. Glucagon-like peptide-1-responsive catecholamine neurons in the area postrema link peripheral glucagon-like peptide-1 with central autonomic control sites. J Neurosci. 2003,23:2939-46.

[33] Baraboi, E. D., Smith, P., Ferguson, A. V., Richard, D. Lesions of area postrema and subfornical organ alter exendin-4-induced brain activation without preventing the hypophagic effect of the GLP-1 receptor agonist. Am J Physiol Regul Integr Comp Physiol. 2010,298:R1098-110.

[34] Marre, M., Shaw, J., Brandle, M., Bebakar, W. M., Kamaruddin, N. A., Strand, J., et al. Liraglutide, a once-daily human GLP-1 analogue, added to a sulphonylurea over 26 weeks

produces greater improvements in glycaemic and weight control compared with adding rosiglitazone or placebo in subjects with Type 2 diabetes (LEAD-1 SU). Diabet Med. 2009,26:268-78.

[35] Calara, F., Taylor, K., Han, J., Zabala, E., Carr, E. M., Wintle, M., et al. A randomized, open-label, crossover study examining the effect of injection site on bioavailability of exenatide (synthetic exendin-4). Clin Ther. 2005,27:210-5.

[36] Spencer, C. M., Eckel, L. A., Nardos, R., Houpt, T. A. Area postrema lesions attenuate LiCl-induced c-Fos expression correlated with conditioned taste aversion learning. Physiol Behav. 2011,105:151-60.

[37] Wang, Y., Lavond, D. G., Chambers, K. C. The effects of cooling the area postrema of male rats on conditioned taste aversions induced by LiC1 and apomorphine. Behav Brain Res. 1997,82:149-58.

[38] Rinaman, L. A functional role for central glucagon-like peptide-1 receptors in lithium chloride-induced anorexia. Am J Physiol Regul Integr Comp Physiol. 1999,277:R1537-40.

[39] Matsukawa, S., Reid, I. A. Role of the area postrema in the modulation of the baroreflex control of heart rate by angiotensin II. Circ Res. 1990,67:1462-73.

[40] Collister, J. P., Nahey, D. B. The cardiovascular response of normal rats to dual lesion of the subfornical organ and area postrema at rest and to chronic losartan. Brain Res. 2009,1302:118-24.

[41] Bello, N. T., Kemm, M. H., Ofeldt, E. M., Moran, T. H. Dose combinations of exendin-4 and salmon calcitonin produce additive and synergistic reductions in food intake in nonhuman primates. Am J Physiol Regul Integr Comp Physiol. 2010,299:R945-52.

[42] de Vente, J., Steinbusch, H. W., Schipper, J. A new approach to immunocytochemistry of 3',5'-cyclic guanosine monophosphate: preparation, specificity, and initial application of a

new antiserum against formaldehyde-fixed 3',5'-cyclic guanosine monophosphate. Neuroscience. 1987,22:361-73.

Figure legends

Figure 1 Baseline 24h food intake (A) and blood levels of amino acids (B) and glucose (C) measured in animals receiving 1%, 8% or 18% protein diet; dotted line represents the average amino acid concentration of animals that received standard chow. Data are expressed as means ± SEM. Different letters indicate significantly differences (A: n=12; B and C: n=4-6; $p < 0.05$; one way ANOVA, Student-Newman-Keuls post hoc test).

Figure 2 Effect of amylin (5µg/kg s.c.) on food intake in rats receiving different test diets containing 1%, 8% or 18% protein (A-C). Bars represent group means ± SEM. (n = 12: 1% and 18%; n = 10: 8 %) *p<0.05, and ***p<0.001 significantly different from respective control (saline) group at individual time points (paired Student t test). D: Food intake of amylin treated rats relative to their respective saline treated controls (=100%)**.** Bars with different letters are significantly different ($p < 0.05$; one way ANOVA on ranks, Dunn' multiple comparison test).

Figure 3 Quantification of the number of c-Fos-IR nuclei in the AP of of rats treated with amylin (5µg/kg s.c.) or saline; all rats were fed ad libitum and received 1% or 18% protein diet, respectively. Data are expressed as means ± SEM (n=7). Bars with different letters are significantly different ($p < 0.05$; one way ANOVA, Student-Newman-Keuls post hoc test).

Figure 4 Effect of Aminoven pre-treatment on the amylin-induced c-Fos expression in the AP of rats; all rats were fasted for 24h fasted in this experiment. Top: representative immunohistochemical c-Fos stainings. Aminoven (1g/kg i.p.), amylin (5µg/kg s.c.); aminoven/saline: n=8; aminoven/amylin: n=7, saline/saline: n=5, saline/amylin: n=7; Scale bar 50µm. Bottom: quantification of c-Fos positive cells; data are expressed as means ± SEM. Significant differences are indicated by different letters ($p < 0.05$; one way ANOVA, Student-Newman-Keuls post hoc test).

Figure 5 Quantification of the number of c-Fos-IR nuclei in the AP of ad libitum fed rats vs fasted rats after GLP-1 (A, 100µg/kg i.p.) or AngII (B, 50µg/kg s.c.). Data are expressed as means ± SEM (n=5). Bars with different letters are significantly different ($p < 0.05$; one way ANOVA, Student-Newman-Keuls post hoc test).

Figure 6 Representative immunostainings and quantification of c-Fos expressing and c-Fos/CTR double positive neurons the AP region of rats fasted for 24h. Treatments: (A-C) amylin (5µg/kg s.c.), (D-F) exendin-4 (2µg/kg i.p.), (G-I) AngII (50µg/kg s.c.); (J) GLP-1 (100µg/kg i.p.), (K) LiCl (76mg/kg i.p.), saline as control for all treatments. White squares: area of higher magnifications shown in (B, E, H). Arrow heads: single labeled neurons (green: c-Fos; red: CTR), arrows: c-Fos/CTR double positive neurons. Scale bar 100µm. Data are expressed as means ± SEM (n=5). Bars with different letters are significantly different ($p < 0.05$; one way ANOVA, Student-Newman-Keuls post hoc test).

Figure legends of supplementary material:

Supplementary figure 1 Quantification of the number of c-Fos-IR nuclei in the AP of ad libitum fed rats and fasted rats after 5 hrs (A) and 1hr (B) of food deprivation. Rats were injected either with amylin (5µg/kg s.c.) or saline. Data are expressed as means ± SEM (n=5). Bars with different letters are significantly different ($p < 0.05$; one way ANOVA, Student-Newman-Keuls post hoc test).

Supplementary figure 2 Representative immunostainings and quantification of cGMP/CTR double positive neurons the AP after amylin treatment (5µg/kg s.c.). (A,B) Co-localization of cGMP and CTR (arrow heads); scale bar 50µm. (C) Numbers of cGMP

and cGMP/CTR positive cells in AP sclices. (D) Percentages of cGMP positive AP neurons that were positive or negative for CTR after amylin treatment.

Figures:

Figure 1

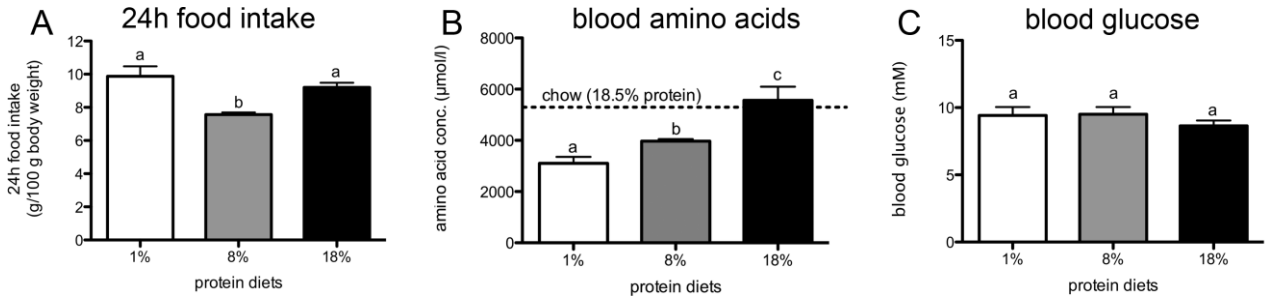


Figure 2

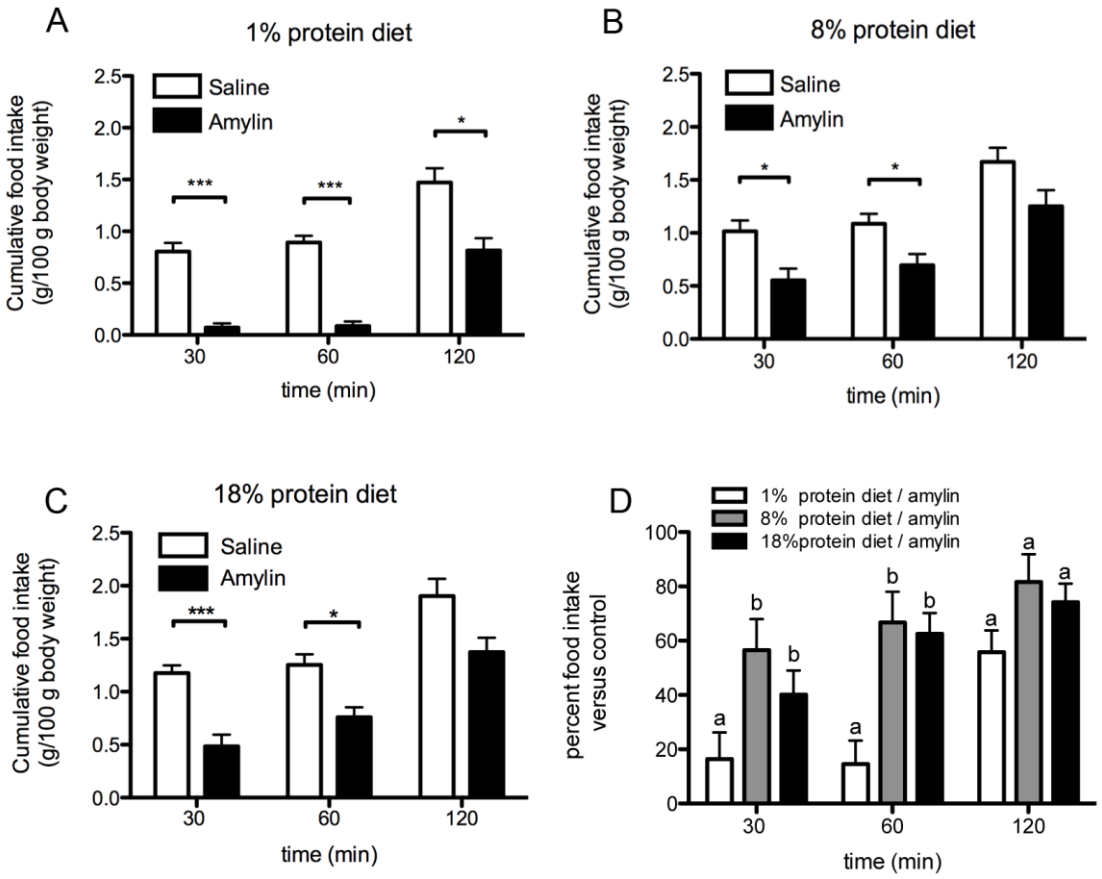

A
1% protein diet
Saline
Amylin
Cumulative food intake (g/100 g body weight)
time (min)
B
8% protein diet
C
18% protein diet
D
1% protein diet / amylin
8% protein diet / amylin
18%protein diet / amylin
percent food intake versus control

Figure 3

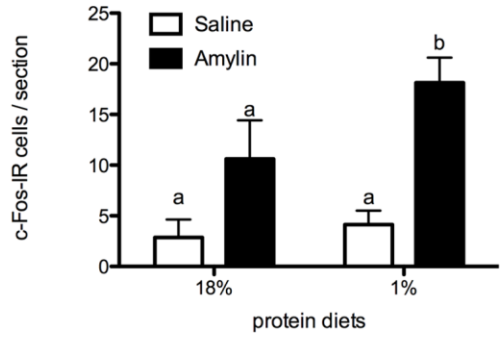

Saline
Amylin
c-Fos-IR cells / section
25
20
15
10
5
0
a
a
a
b
18%
1%
protein diets

Figure 4

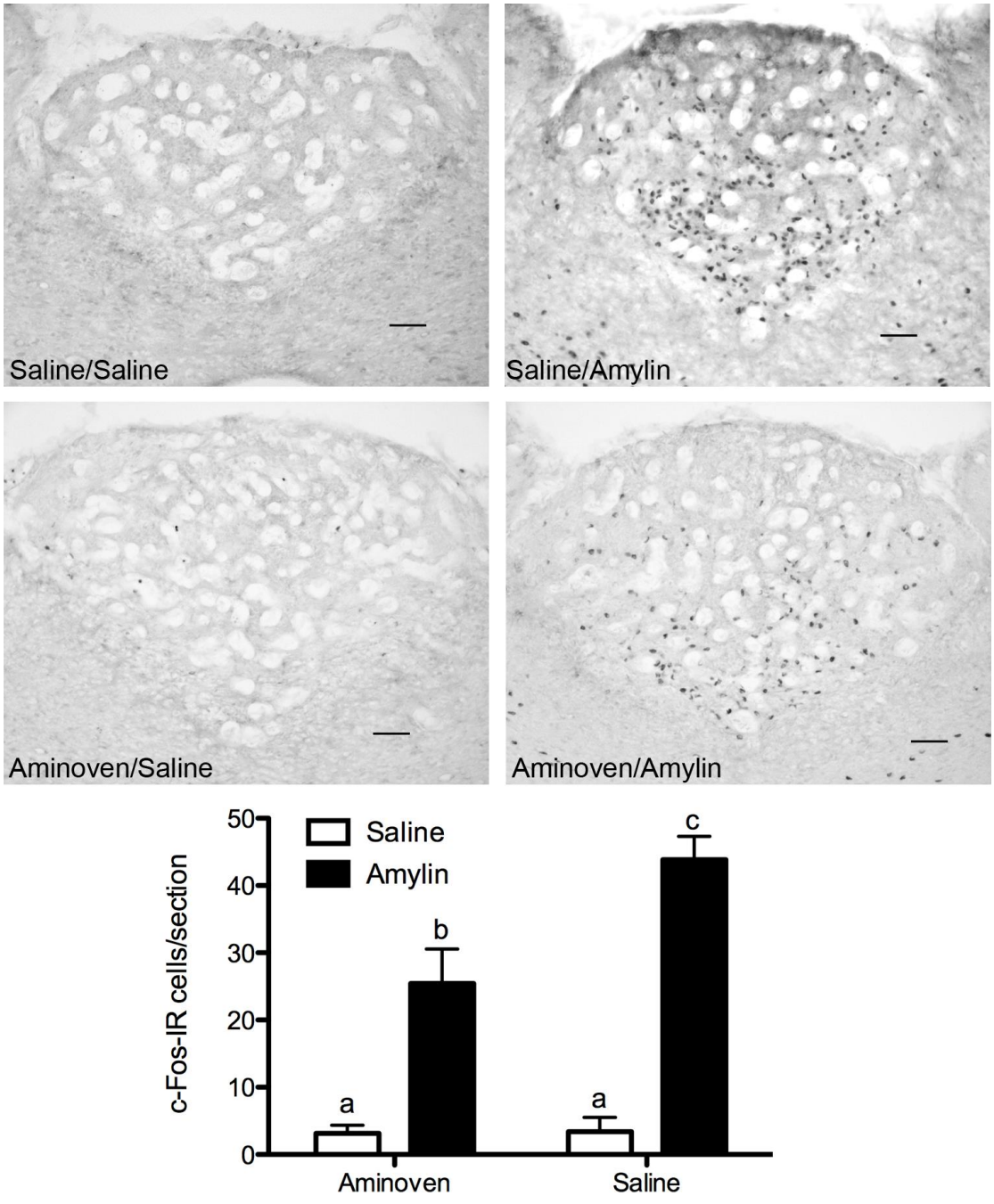

Saline/Saline
Saline/Amylin
Aminoven/Saline
Aminoven/Amylin
c-Fos-IR cells/section
50
40
30
20
10
0
Saline
Amylin
a
b
a
c
Aminoven
Saline

Figure 5

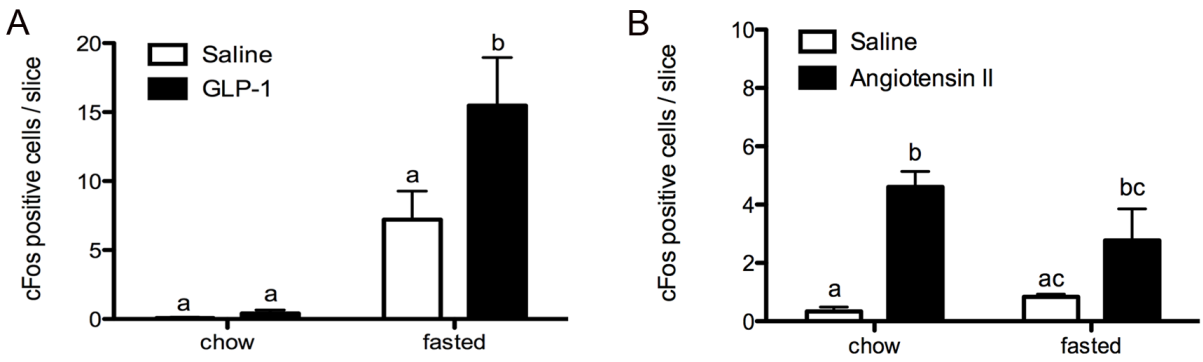
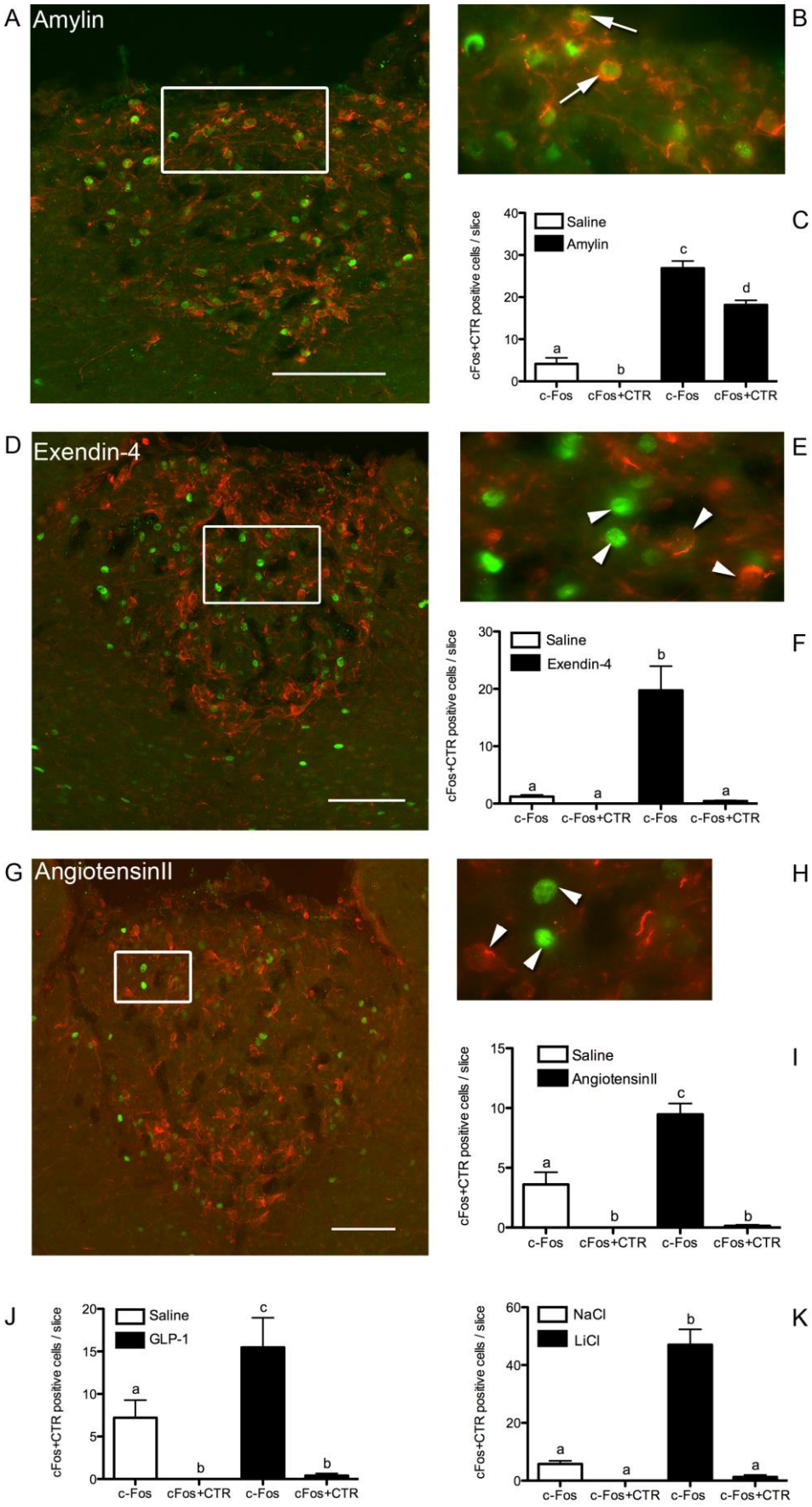
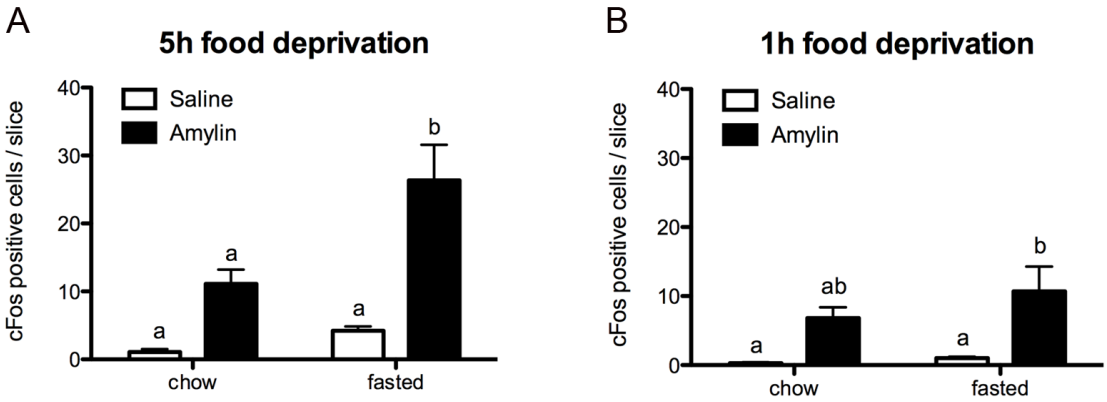

A
cFos positive cells / slice
20
15
10
5
0
Saline
GLP-1
a
a
a
b
chow
fasted
B
cFos positive cells / slice
10
8
6
4
2
0
Saline
Angiotensin II
a
b
ac
bc
chow
fasted

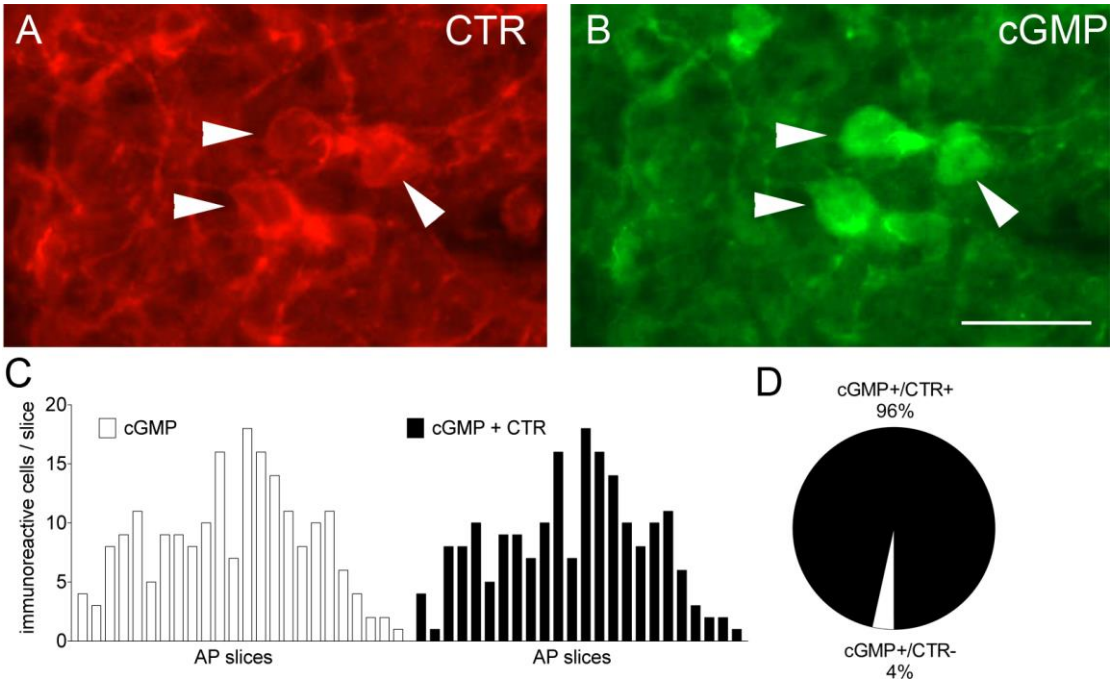
Figure 6


A
Amylin
B
C
Saline
Amylin
cFos+CTR positive cells / slice
c-Fos
cFos+CTR
D
Exendin-4
E
F
Saline
Exendin-4
G
AngiotensinII
H
I
J
Saline
GLP-1
K
NaCl
LiCl

Supplementary figure 1



Supplementary figure 2



Acknowlegements

I would like to thank everybody who supported my work within the last years.

Especially warm thanks go to

Professor Dr. Thomas A. Lutz for giving me the opportunity of working in his group.

PD Dr. Thomas Riediger for the great supervision, the help with writing and proof reading this manuscript.

Special thanks go to

Sara, Lette and Josi for the care of animals.

Christina, Melania, Catharina, Tito, Daniela M., Daria, Nadine, Lori, Miriam, Melanie, Karoline, Sarah, Kerstin for the daily office life and help.

Last but not least I would like to thank **Martin** for always being there and gave me the strength to finish this work.